THE CHLORIDE ION IN THE HEMOLYMPH OF THE LARGE MILKWEED BUG, ONCOPELTUS FASCIATUS (DALLAS) [1, 2]

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Although the literature revealed some figures on the chloride ion content of the hemolymph of a few insects, only one adult insect, *Dytiscus marginalis* L., has been so studied. Most workers bled a great many insects to obtain a sufficiently large sample or chose a larva or pupa of a species with complete metamorphosis relatively rich in hemolymph. The following table indicates the species, life stage, chloride in milligrams per 100 ml. and the investigator for all such references:

TABLE 1
Chloride Determinations in Hemolymph from the Literature

Species of Insect	Stage	Cl- mg./ 100 ml.	Investigator
Prodenia eridania (Cramer)	larva	119.8	Babers (1938)
Apis mellifera Linné	larva	117.0	Bishop, Briggs, and Ronzoni (1925)
Sphinx pinastri Linné	pupa	65.9 ♂ 58.1 ♀	Brecher (1929)
Deilephila euphorbiæ Linné	larva	48.6	Heller and Moklowska (1930)
Dytiscus marginalis Linné	adult	224.0	Portier and Duval (1927)
Saturnia carpini Schiffner	larva	42.5	Portier and Duval (1927)
Cossus cossus Linné	larva	6.69	Portier and Duval (1927)
Bombyx mori Linné	larva	51.6	Portier and Duval (1927)
Bombyx rubi Fabricius	larva	89.8	Portier and Duval (1927)
Sphinx ligustri Linné	pupa	53.4	Portier and Duval (1927)
Saturnia pyri Schiffner	pupa	62.5	Portier and Duval (1927)
Aedes ægypti (Linné)	larva	182.0	Wigglesworth (1938)
Culex pipiens Linné	larva	170.0	Wigglesworth (1938)

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Of the six papers treating of an analysis of insect hemolymph for chloride, two, Heller and Moklowska (1930) and Portier and Duval (1927) did not give the method of analysis. The rest, including Wigglesworth's (1937), were all special modifications of the Volhard determination of chlorides, a method dependent on a colorimetric end point.

The method used involved a potentiometric determination of the end point. Basically this method was first proposed by Cunningham, Kirk, and Brooks (1941). In this present study there were several modifications of the original methods, the most important being the substitution of a vacuum-tube voltmeter for the potentiometer.

APPARATUS AND TECHNIQUES

1. ELECTRODES. A potentiometric method utilizing a bimetallic system of electrodes was used. One electrode was of Number 23 silver wire soldered to a piece of Number 14 copper wire to afford a good connection with the electrode holder. This electrode was the indicator electrode. A small bulb was fashioned in the end which dipped into the solution to furnish a good surface for contact with the solution. The other electrode, which was of Number 23 tungsten wire, served merely to complete the circuit. Both electrodes were thoroughly cleaned with fine emery paper at the beginning of a series of titrations to remove any deposit of silver chloride. It was found that this had the same effect on the silver electrode as dipping it in a solution of nitric acid with a small amount of sodium nitrite added until effervescence occurred as recommended by Cunningham, Kirk, and Brooks (1941).

2. VACUUM-TUBE VOLTMETER. An instrument of this kind was found to be more convenient than a potentiometer in determining the difference in potential across the electrodes, because once set there is no manipulation needed to determine the individual readings, because the latter are read directly from a meter. Furthermore, the titration curve can be obtained more accurately in a shorter period of time than by the use of a potentiometer, because after each addition of silver nitrate a shorter or longer period of

time elapses before equilibrium is reached. On a vacuum-tube the needle continues to swing back and forth slowly until an equilibrium for that addition of silver nitrate is reached.

The vacuum-tube voltmeter was constructed from a diagram given in "Radio Test Instruments" by R. P. Turner. The only change which was made was the use of a d'Arsonval galvanometer instead of the 0–1 D. C. milliammeter suggested. The latter is not sensitive enough to record the end point satisfactorily in all titrations, since the magnitude of the break in potential may be greater or lesser in several titrations, the important point being that it corresponds with the equivalence point.

3. Burette. The burette, manufactured by Microchemical Specialties in Berkeley, California, had a total capacity of 0–1 ml. which was divided into 350 divisions. Therefore, additions could be made as small as 0.286 microliters without estimations. Readings could be estimated to tenths of each division. The burette was calibrated by the company and was similar to the one described by Cunningham, Kirk, and Brooks (1941).

4. Stirrer. The solution being titrated was stirred rapidly by means of a fine glass rod cemented by rosin to a copper wire which in turn was soldered to the vibrator of an electric buzzer. The speed of vibration was controlled by a rheostat inserted into the circuit, the speed being cut down to the point where it did not cause splashing of the solution.

5. Collecting of hemolymph. Although some workers have stated that the hemolymph may be easily collected by cutting the legs or by making a dorsal incision and allowing the hemolymph to drip into a test tube, I have not found this to be true with the large milkweed bug. A small droplet of hemolymph did appear when an incision was made. However, this did not "drip" of its own accord. It was necessary to touch the edge of the vessel to the droplet in order to secure the hemolymph. A microvial which is generally used in taxonomic studies, was employed to collect the fluid. Since the hemolymph of the milkweed bug does not clot, the insect did not have to be first dipped in hot water or treated with acetic acid. In all cases the legs were cut off approximately midway along the femur and the slightly yellow clear drops of

hemolymph which formed were touched with the collecting vial. A small amount of pressure was placed on the abdomen to force out an additional amount of hemolymph. However, care was exercised in forebearing from collecting any fluid which appeared turbid or in any way contaminated with another tissue. Precautions were also taken to avoid contaminating the collected hemolymph with sodium chloride from the hands. The collecting vial was washed in distilled water and dried inside and out with fresh cotton before use and handled only with forceps. When actually in use, it was inserted into a glass tube filled almost to the top with paraffin. In this way one could hold the glass tube instead of attempting to maintain the proper tension with forceps. About five adults, sometimes one or two more for males and sometimes one or two less for females were needed to collect an adequate amount to furnish a sample of 7.58 microliters. Approximately eight nymphs of the fifth instar and fifteen nymphs of the fourth instar were required to obtain a volume of the same size. In order to obtain 3.98 microliters in the neighborhood of twenty-five to thirty individuals of the third, second and first instar had to be bled. About ten minutes were required to bleed the adults; approximately twenty minutes to bleed the fifth and fourth instars, and over thirty minutes for the third, second and first. When a suitable amount was obtained, the hemolymph was taken up with a calibrated pipette. The pipette could not be used directly without the use of the collecting vial, because air tended to be introduced with a subsequent misconception of volume.

6. PIPETTES. The pipettes were made of soft glass with a fine point on one end to insert into the collecting vial, a rounded bulb to contain the bulk of the sample, and a constricted area where the capacity mark was made with a diamond marking pencil. This was similar to an ordinary pipette except that it was made on a smaller scale and it was in this respect that it differed from the pipette employed by Cunningham, Kirk, and Brooks (1941) from whose work the remainder of the pipette was designed. After the constriction, the glass widened out. Into this area a Number 27 syringe needle was inserted and glued by means of rosin. A 0.1 ml. syringe was then inserted into the needle of this pipette to

force hemolymph in and out of the measuring chamber. When a syringe having a larger bore was used, the vacuum created was too great and the hemolymph was moved past the capacity mark into the needle of the syringe.

The pipettes were calibrated by weighing the amount of mercury delivered on an analytical balance.

KNOWN CONCENTRATIONS OF CHLORIDE

The method was tested for accuracy by titrating various concentrations of silver nitrate against known amounts of sodium chloride solutions, calculating on the basis of chloride ion content rather than on the salt concentration. Using analytical grade chemicals a 0.1 M solution of sodium chloride and a 0.1 M solution of silver nitrate were made up as primary standards by the use of approved methods. Just before use these were carefully diluted to the necessary concentration. Table 2 shows the titrations of various concentrations of sodium chloride with various concentrations of silver nitrate together with the accuracy thereof.

In order to determine the applicability of the method to hemolymph two samples of adult hemolymph were taken. One was analyzed directly in the described manner. To the other a small quantity of sodium bicarbonate was added. The sample was then ashed. The ash was taken up in 40 microliters of distilled water and subjected to analysis in the same manner as the first sample. The results of the two determinations were practically the same.

HEMOLYMPH OF ONCOPELTUS FASCIATUS (DALLAS)

Adults of various ages, males, and females, and all five instars of the nymphs were analyzed for chloride content of the hemolymph. The molarity of the silver nitrate used for the adults through the fourth instar nymphs was 0.005; for the first three instars, 0.001. The results are shown in Table 3. The plasma of adults and of the fifth instar nymphs was also analyzed, the results also appearing in Table 3.

TABLE 2

TITRATIONS OF KNOWN CONCENTRATIONS OF CHLORIDE

Concentration Cl- Micrograms/ Microliter	Molarity $AgNO_3$	Units $AgNO_3$ Required	Amt. Cl- Calculated (Micrograms)	Amt. Cl- Found (Micrograms)	Deviation (Micrograms)	Mean Deviation	Per Cent Error	Mean Recovery Per Cent	Standard Deviation (0′)	Standard Error of Standard Deviation
0.7092	0.1M	3.24	35.46	35.39	0.07					
		3.25		35.49	0.03					
		3.25		35.49	0.03					
		3.24		35.39	0.07					
		3.25		35.49	0.03	± 0.046	± 0.13	99.97	0.559	0.174
0.7092	0.05	6.54	35.46	35.48	0.02					
		6.53		35.42	0.04					
		6.53		35.42	0.04					
		6.52		35.37	0.09					
		6.55		35.53	0.07	± 0.052	± 0.15	99.94	0.644	0.204
0.7092	0.01	33.79	35.46	35.47	0.01					
		33.78		35.46	0.00					
		33.77		35.45	0.01					
		33.78		35.46	0.00					
		33.76		35.44	0.02	± 0.008	± 0.023	100.00	0.122	0.0386
0.3546	0.05	3.25	17.73	17.74	0.01					
		3.26		17.80	0.07					
		3.24		17.69	0.04					
		3.24		17.69	0.04					
		3.25		17.74	0.01	± 0.034	± 0.19	100.00	0.455	0.144
0.3546	0.01	16.56	17.73	17.73	0.00					
		16.55		17.72	0.01					
		16.56		17.73	0.00					
		16.54		17.71	0.02					
		16.55		17.72	0.01	± 0.008	± 0.045	99.94	0.122	0.0386
0.3546	0.005	33.79	17.73	17.73	0.00					
		33.81		17.74	0.01					
		33.78		17.73	0.00					

TABLE 2 (*Continued*)

Concentration Cl- Micrograms/ Microliter	Molarity $AgNO_3$	Units $AgNO_3$ Required	Amt. Cl- Calculated (Micrograms)	Amt. Cl- Found (Micrograms)	Deviation (Micrograms)	Mean Deviation	Per Cent Error	Mean Recovery Per Cent	Standard Deviation (0′)	Standard Error of Standard Deviation
0.07092	0.01	3.26	3.546	3.560	0.014					
		3.25		3.549	0.003					
		3.24		3.539	0.007					
		3.24		3.539	0.007					
		3.25		3.549	0.003	± 0.0068	± 0.19	100.03	0.279	0.0883
0.07092	0.005	6.54	3.546	3.548	0.002					
		6.56		3.559	0.013					
		6.53		3.542	0.004					
		6.55		3.553	0.007					
		6.53		3.542	0.004	± 0.006	± 0.17	100.08	0.252	0.0797
0.07092	0.001	33.77	3.546	3.545	0.001					
		33.79		3.547	0.001					
		33.78		3.546	0.000					
		33.80		3.548	0.002					
		33.76		3.544	0.002	± 0.0008	± 0.023	100.00	0.050	0.0158
0.03546	0.001	16.56	1.773	1.773	0.000					
		16.53		1.770	0.003					
		16.57		1.774	00.01					
		16.54		1.771	0.002					
		16.53		1.770	0.003	± 0.0018	± 0.10	99.94	0.0758	0.0237
0.03546	0.0005	33.75	1.773	1.771	0.002					
		33.73		1.770	0.003					
		33.82		1.774	0.001					
		33.85		1.776	0.003					
		33.73		1.770	0.003	± 0.0024	± 0.14	99.94	0.0894	0.0283

TABLE 3

	No. of Samples	Mean Amt. Cl - Milligrams/ 100 Ml.	Mean Deviation	Standard Deviation	Standard Error of Standard Deviation
Chloride in Whole Hemolymph of Adults—Male and Female	25	91.4	± 0.114	0.0689	0.00975
Chloride in Whole Hemolymph of Male Adults	10	91.2	± 0.007	0.138	0.0309
Chloride in Whole Hemolymph of Female Adults	10	91.1	± 0.012	0.141	0.0315
Chloride in Whole Hemolymph of Fifth Instar Nymphs	10	91.5	± 0.026	0.332	0.0743
Chloride in Whole Hemolymph of Fourth Instar Nymphs	10	91.5	± 0.027	0.336	0.0751
Chloride in Whole Hemolymph of Third Instar Nymphs	10	91.4	± 0.009	0.257	0.0575
Chloride in Whole Hemolymph of Second Instar Nymphs	10	91.2	± 0.008	0.367	0.0821
Chloride in Whole Hemolymph of First Instar Nymphs	10	91.2	± 0.007	0.202	0.0452
Chloride in Plasma of Adults	10	142.9	± 0.008	0.494	0.111
Chloride in Plasma of Fifth Instar Nymphs	10	143.0	± 0.009	0.496	0.111

CONCLUSIONS

1. A potentiometric method, utilizing a bimetallic system of electrodes and a vacuum-tube voltmeter was established for measuring the chloride ion content of a microsample of hemolymph with an error of not more than 0.2 per cent down to 0.03546 micrograms of chloride per microliter.

2. The chloride ion content of the hemolymph of the large milkweed bug, *Oncopeltus fasciatus* (Dallas), was found to be 6.93

micrograms per 7.58 microliters or 91.4 milligrams per 100 milliliters.

3. No difference was determined in the chloride content of the hemolymph between the sexes or between the adults and any one of the five instars of nymphs.

4. The chloride ion content of the plasma alone was found to be 10.83 micrograms per 7.58 microliters or 143 milligrams per 100 milliliters.

5. There was no difference in the chloride content of the plasma between the adults and fifth instar nymphs.

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